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Mitochondrial Heterogeneity in *Aspergillus nidulans: in vivo* Protein Biosynthetic Activities of the Mitochondrial Populations

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Two mitochondrial populations with cytochrome c oxidase activity, and densities of 1.07 and 1.08 g/ml have been separated by means of a dextran-sucrose gradient. The highest enzymatic specific activity appeared in the 1.07 g/ml band. The mitochondria assayed by their *in vivo* cycloheximide resistant leucine incorporation appeared at the 1.08 g/ml density band of the gradient. Some amount of activity of cytochrome c oxidase, and leucine incorporation appeared at the bottom of the gradient. The cytochrome spectra in liquid nitrogen of each band showed different proportion of cytochrome(s) a, b and c. The highest ratio of cytochrome a and cytochrome b in respect to citochrome c appeared in the 1.08 g/ml band.

The isolation of different mitochondrial populations has been repeatedly described (3, 6, 7, 10, 11, 13) specially from rat liver where two populations have been separated by means of sucrose gradients (9-11). Different mitochondrial populations from yeast have also been separated in sucrose gradients (1, 7) but mitochondrial heterogeneity in fungi has been less studied than in animal cells. Isotonic gradient generated by macromolecules such as Ficoll or Dextran T 40 and isoosmotic D_2O gradients have been also used to study this mitochondrial heterogeneity (8, 16-18).

The presence of at least two mitochondrial populations in *Aspergillus nidulans* has been previously described. Pulse chese experiments with ^{3}H-glycerol suggested the existence of a relationship between this heterogeneity and different biogenetic stages of the mitochondria (5).

This paper shows further evidences of the occurrence of this heterogeneity as characterized by enzymatic and cytochrome spectra as well as the existence of a population which *in vivo* selectively incorporates leucine by a cycloheximide resistant process.

Materials and Methods

Organism. The strain of *Aspergillus nidulans* used in this work was the R-21 paba A_1, yA_2 (a p-amino benzoic acid auxotrophic); kindly supplied to us by Dr. Turner of the University of Bristol. Conidia and mycellium were grown in the medium previously described (12), with glucose as the source of carbon. The strain was cultured in 1,500 ml minimal medium in a conical flask in an orbital incubator (Gallenkamp) at 180 r.p.m. and at 37° C for 20 hours. The media contained the required auxotrophic supplements and the concentration of conidia added was 10^6/ml.

Preparation and fractionation of mitochondria. Mitochondria were extracted as described by TURNER (19) and fractionation on a dextran-sucrose was carried out as previously described (5).

Cytochrome c oxidase activity. Cytochrome c oxidase was tested by following reoxidation of cytochrome c, reduced with sodium dithionite, as previously described by TURNER (19). Results are expressed in μmol of cytochrome c oxidized $\times$ min^{-1}.

Counting of radioactivity. 0.5 ml of each gradient fraction were precipitated with 0.5 ml of 20 % trichloroacetic acid, filtered on Watman GF/C filters and washed with 5 ml of 5 % trichloroacetic acid. Dried samples were counted in 2 ml of scintillation liquid (1,4-bis-2-(4-methyl-6-phenyloxazolyl)-benzol 0.2 g; 2,5-diphenyl-oxazole 10 g, toluene 2 l and Triton X-100 1 l) in a Packard Tri-Card mod. 2425 scintillation counter with a 63 % efficiency.

Cytochrome spectra. Spectra of the isolated mitochondrial populations were examined at 180° C (in liquid N_2) in a split beam spectrophotometer (2) with a slit of 0.15 mm. The cuvettes (path length, 2 mm) contained 0.6 ml of suspension.

Results

Isolation of mitochondrial populations. Figure 1 a shows the distribution of the cytochrome c oxidase activity obtained when a crude mitochondrial pellet was centrifuged in a dextran-sucrose gradient. The enzymatic activity appeared in three different positions along the gradient. The highest proportion of the activity was present at the light side of the gradient. The second peak was found sedimented at middle density and, finally some activity appeared at the bottom of the tube, on the 1.7 M sucrose cushion.

The sedimentation of the light and middle band occurred in fractions with a refraction index of 1.368 $\pm$ 0.003 and 1.375 $\pm$ 0.003 corresponding to densities of 1.07 and 1.08 g/ml respectively. Similar results have been previously described (5).

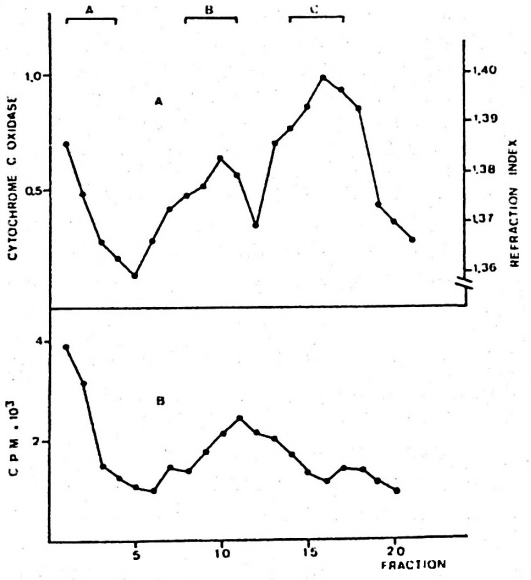

Fig. 1. *Centrifugation on a dextran-sucrose gradient of* Aspergillus nidulans *mitochondria. a*) Cytochrome c oxidase activity expressed as μmol $\times$ min^{-1} $\times$ ml^{-1} (●—●); Refraction index (O—O); *b*) Cycloheximide resistant incorporation of ^{3}H-leucine expressed in c. p.m. $\times$ ml^{-1}.

Effect of cycloheximide in the in vivo *incorporation of ^{3}H-leucine in the mitochondrial fractions.* 20 hours mycelium was grown for further 10 minutes in the presence of 100 μg/ml of cycloheximide. Then 0.07 μCi/ml of ^{3}H-leucine (specific activity of 53 mCi/mmol) was added to the mycelium and allowed to grow for 30 minutes with the isotope. After the alotted time it was collected and washed with the growth medium. Mitochondrial fractions were prepared as described in «methods» and sedimented in a dextran-sucrose gradient. It can be observed (figure 1 b) that the highest incorporation appeared at the band which sedimented at the middle side of the gradient (refraction index of 1,375).

Cytochrome spectra of isolated mitochondria. Figure 2 a, b and c show the low temperature spectra (dithionite reduced minus oxidized) of the sedimentation bands of a dextran-sucrose gradient.

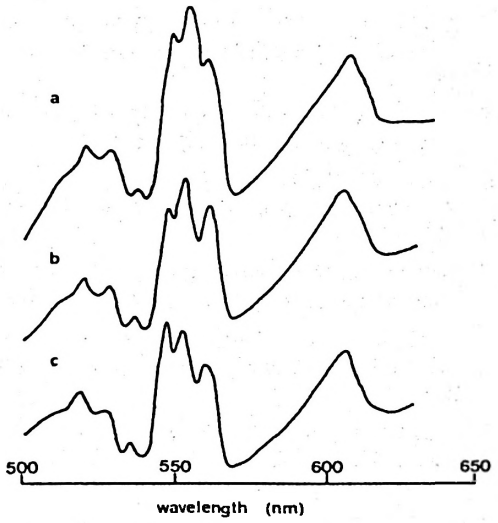

Fig. 2. *Low temperature spectra (dithionite-reduced minus oxidized) of mitochondria from the three zones showed in figure 1.* *a*) Zone A; *b*) Zone B; *c*) Zone c.

Table I. *Specific activities of cytochrome c oxidase leucine incorporation, and cytochrome a + cytochrome b/ cytochrome c ratio of the different zones of figure 1.*

Zone	Density g × ml^{-1}	Cytochrome c oxidase μmol × min^{-1} × mg^{-1}	^{3}H-leucine c.p.m. × mg^{-1}	Cyt. a + Cyt. b/ Cyt. c $E_{607}+E_{555}/E_{547.5}$
A	1.24	0.865	385.2	1.31
B	1.08	1.269	1,232.0	1.93
C	1.07	1.360	981.5	1.73

Cytochrome a. a_3 was responsible for the α-bands at 607 nm, cytochrome b was resolved into two α-bands at 555 and 564 nm and the α-band of cytochrome c was visible at 549.5 nm the β-band of cytochrome c was visible at 521 nm. The remaining two bands were probably due to cytochrome (s) b.

Quantification of the spectra was carried out by measuring the differences of the optical densities between the maximun of the α-bands and the base line traced among the minimum at 630, 570 and 540 nm. Table I shows the ratio of cytochrome a and cytochrome b in respect to the cytochrome c as well as the specific enzymatic activities of cytochrome c oxidase and leucine incorporation of each band. It can be observed that the highest values for cytochrome c oxidase appeared at the lightest band, whilst cytochrome a + cytochrome b/ cytochrome c ratio and leucine incorporation was higher at the middle band.

Discussion

The *in vivo* cycloheximide resistant leucine incorporation was carried out in order to find out whether any of the populations isolated on dextran-sucrose gradient was more active in their proper protein synthesis. Several authors (14, 15, 20) have proven that at least part of the

cycloheximide resistant synthesis is due to mitochondrial protein synthesis. TURNER (19) showed that the labelling appeared preferentially in mitochondrial proteins. The middle density mitochondria were the most active in incorporating leucine in the presence of cycloheximide. Similar results were obtained with Hela cells mitochondria (17).

The cytochrome spectra reported (fig. 2) was similar to those obtained by TURNER and ROWLANDS (20) in *Aspergillus nidulands*, although in our results cytochrome b has only been resolved in two α-bands (555 and 564 nm). A different proportion of cytochrome a, b and c was observed in the three populations. An increase in the proportion of cytochrome b (564 nm α-band) and cytochrome a (607 nm α-band) is clearly visible in the middle band. An approximate quantification of these results is represented in table I, which shows that the highest proportion of cytochrome a+cytochrome b/ cytochrome c was present in the middle density mitochondria and the smallest one appeared in the light population. Since it is usually assumed that some subunits of the cytochrome a and b are synthesized by mitochondrial ribosomes and cytochrome c by cytoplasmic ones (4), the cytochrome spectrum of these middle density mitochondria (with the highest cytochrome a + cytochrome b/ cytochrome c ratio) is in agreement with the suggestion that this population is the most active one in mitochondrial protein synthesis.

The nature of the band at the bottom of the tube remains unknown to us. A similar band with cytochrome c oxidase activity which showed cycloheximide resistant leucine incorporation, with high contamination of membranes, has also been described by STORRIE and ATTARDI (17). We cannot discard the possibility that the contamination in our results might be due to membranes of disrupted mitochondria. This may explain the high values found in this band.

Resumen

Se han separado de *A. nidulans* en gradiente de dextrano-sacarosa dos poblaciones mitocondriales con actividad citocromo c oxidasa, de densidades 1,07 y 1,08 g/ml, apareciendo la mayor actividad enzimática específica en la banda de 1,07 g/ml. Por el contrario, la banda de 1,08 g/ml es la que incorpora *in vivo* leucina en presencia de cicloheximida. Los espectros de citocromos en nitrógeno líquido de cada banda muestran diferentes proporciones de citocromo(s) a, b y c. La relación mayor entre citocromo a y citocromo b respecto a citocromo c aparece en la banda de 1,08 g/ml.

References

1. CARTLEDGE, T. G. and LLOYD, D.: *Biochem. J.*, **126**, 381-396, 1972.
2. JONES, O. T. G. and SAUNDERS, V. A.: *Biochim. Biophys. Acta*, 275, 427-436, 1972.
3. KATYARE, S. S., FATTERPAKER, P. and SREENIVASAN, A.: *Biochem. J.*, 118, 111-121, 1970.
4. LLOYD, D.: «The mitochondria of microorganism». Academic Press, London, 1974, pp. 420-428.
5. MONTOYA, J. and LÓPEZ-PÉREZ, M. J.: *Rev. esp. Fisiol.*, **36**, 27-32, 1980.
6. NEAL, W. K., HOFFMANN, H. P., PRICE, C. A.: *Biochem. Biophys. Res. Com.*, **38**, 414-423, 1970.
7. NEIDLE, A. VAN DER BESP, C. J. and GRYNBAUN, A.: *J. Neurochem.*, **15**, 225-233, 1969.
8. PICKETT, B. B., CASCARANO, J. and JOHNSON, P.: *J. Bioenerg. Biomembr.*, **9**, 271-282, 1977.
9. POLLAK, Y. K.: *Biochem. Biophys. Res. Com.*, **69**, 623-829, 1976.
10. POLLAK, J. K.: *Biochem. Soc. Trans.*, **5**, 241-248, 1977.
11. POLLAK, J. K. and MUNN, E. A.: *Biochem. J.*, **117**, 913-919, 1970.
12. ROWLANDS, R. T. and TURNER, G.: *Mol. Gen. Genet.*, **126**, 201-216, 1973.